Rapid and Sensitive Bioanalytical Method Development and Validation for Quantification of Metoprolol Using LC-MS/MS in Human Plasma

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Summary: A novel, accurate, simple and selective LC-MS/MS method was developed and validated for the determination of metoprolol in human plasma. Due to structural resemblance Propranolol was selected as internal standard. Anti coagulant used was K2 EDTA. Metoprolol, used in the therapy and management of hypertension, myocardial infraction and other cardio vascular diseases. Liquid — liquid extraction technique with tert-butyl methyl ether was applied for the extraction of analyte from human plasma. Kromasil C18 column (5µ, 100 x 4.6 mm) with an isocratic mobile phase of 5mM Ammonium Formate pH 3.5 and Acetonitrile (15:85% V/V) was used for the resolution. Sample ionization was done with Electrospray ionization technique in positive ion mode. Selectivity was enhanced by tandem mass spectrometric analysis via two multiple reaction monitoring (MRM) transitions, m/z 268.15→115.90 for metoprolol and 260.17→115.90 for Propranolol respectively. The linearity of the method was established over a concentration range of 1.505 – 538.254 ng/mL, in human plasma, with the precision and accuracy ranging from 4.67 to 7.41% and 90.66 to 98.15% respectively. The stability of the analyte was evaluated in plasma under different storage conditions.

Key Words: Metoprolol, LC-MS/MS, Kromasil, Electrospray, K2 EDTA, Human Plasma.

Introduction

Metoprolol (MPL), a cardio selective β1-adrenoreceptor antagonist, used in the treatment of various cardiovascular disorders. MPL is available in various salt forms and dosage forms like tablets, capsules and parenterals. [1] Metoprolol (Fig 1 A) is chemically described as 1-[4-(2-methoxyethyl)phenoxy]-3-(propan-2-ylamino)propan-2-ol. Its empirical formula is C25H32N2O2. The molecular weight of MPL is 267.37 g/mol.

Besides the availability of various conventional dosage forms of MPL, research on novel formulations have been increasing day by day viz., pulsatile drug delivery systems [2] and pellets [3] etc. To evaluate the bioequivalence, pharmacokinetic profiles of MPL in different dosage forms plays a vital role for pharmaceutical and clinical research.

Several methods have been reported in the literature for the estimation of MPL and its metabolites in human plasma like Gas chromatography (GC) [4-7] and HPLC with UV detector [8, 9], fluorescence detector [10-13], Mass detector [15-18]. MPL from human plasma was extracted on solid phase [13] and liquid-liquid extraction techniques with dichloro methane [4, 10, 12, 14], n-butyl chloride [13] and mixture of dichloromethane and diethyl ether [4, 10]. After extraction of plasma metoprolol or in urine by solid or liquid-liquid phase extraction, the fluorescence detection used in most methods. While fluorescence detection [10-13] is generally a sensitive and relatively sensitive detection technique Selective for HPLC, they achieve a higher degree of selectivity using tandem mass spectrometry (MS). Metabolites, other drugs or endogenous compounds generally have a different mass / charge ratio (m/z) of the analyte of interest. This reduces the risk of interference and can also improve sensitivity.

Normal phase solvents such as diethyl ether [4] are generally incompatible with Electrospray (ESI) and atmospheric pressure ionization (APCI) of mass spectrometers, because they impose a risk of explosion in the presence of high voltage of the needle for electrospray used. In ESI or in the presence of the corona discharge used in APCI. Therefore, it is better to avoid these solvents unless safety measures are taken, such as the broad post-column addition of MS-compatible mobile phases.

Existing methods for metoprolol separation in human plasma are generally based on normal phase modes [8, 9, 13] and are therefore not transferable to MS detection. Another serious problem with the methods reported was multiple extraction [11, 13, 14] steps, which makes the method laborious and tedious. By keeping in view all the problems, an attempt has been made to develop a simple and reproducible method for the determination of MPL in human plasma by LC-MS/MS using internal standard (IS) as Propranolol. USFDA guidelines were followed for the validation.

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Experimental

Chemicals and reagents

The reference sample of Metoprolol (99.90%) and IS, Propranolol were gifted by Hetero labs, Hyderabad, India. Milli-Q Water used for LC-MS/MS analysis. HPLC grade Methanol and Acetonitrile was purchased from Merck, Mumbai, India. All other chemicals were of analytical grade purchased from SD Fine Chem, Mumbai, India. K2-EDTA human plasma was procured from Santhiram Medical College and General hospital, Nandyal, India.

Instrumentation and chromatographic conditions

Alliance 2695 system (Waters Corporation, Milford, USA) equipped with quaternary pump, solvent degasser, auto sampler and column over was used. Kromasil C18 column (5µ, 100 × 4.6 mm) with a protecting guard column (5µ, 5 x 2.1 mm) of the same type was used, maintained at 30°C. 5mM Ammonium Formate pH 3.5 and Acetonitrile (15:85 % V/V) was used as mobile phase. The flow rate of mobile phase was kept at 1.00 mL/min. 15°C was maintained in auto sampler with 3000 psi pressure. Detection of the analyte and internal standard was performed on a high-resolution accurate Quatro Premier XE triple quadrupole mass spectrometer equipped with ESCI multi-mode ionization source. The mass spectrometer operated in positive electrospray ionization mode (ESI+) with the following conditions: capillary voltage 3.25 kV; source and desolation temperature 285°C and 480°C respectively. Acquisition was performed in Multiple Reaction Monitoring (MRM) mode, monitoring two specific transitions for analyte and IS with a dwell time of 100 ms. The analyte – dependent MS/MS parameters were optimized via direct infusion of tuning standard solution into mass spectrometer. Data analysis was performed using MassLynx 4.1 Software (Waters Corporation, Milford, USA).

Preparation of standards:

Metoprolol stock solution (1 mg/mL) and internal standard Propranolol were prepared separately in a 10 mL volumetric flask dissolved in acetonitrile. The stock solution of IS (Propranolol) was diluted with water: acetonitrile to get the final working standard solution of 0.5 µg/mL. The working standard solution of Metoprolol (50µL) was added to 950 µL drug free plasma and prepared MPL concentration levels of 1.505, 3.009, 5.787, 14.468, 28.937, 57.873, 137.793, 275.586, 430.604 and 538.254 ng/mL.

Quality control samples:

MPL theoretical concentrations in plasma samples at 1.505 ng/mL, 4.492 ng/mL, 112.310 ng/mL, 233.978 ng/mL and 417.819 ng/mL were considered to be appropriate as Lower limit of Quantification (LLOQ), Low Quality Control (LQC), Medium Quality Control (MQC 1 and MQC 2) and High Quality Control (HQC) respectively to validate the bioanalytical method. All solutions were stored at 4°C.

Sample Preparation

A volume of 200 µl of plasma was transferred into a vial, then 50 µl of IS standard solution (2 ng / ml) were added and 100 µl of 2% formic acid were added and stirred for 10 minutes. 2.5 ml of methyl tert-butyl ether were added and centrifuged at 2500 RPM for 10 minutes, the organic layer was transferred to a new vial and evaporated to dryness under a light stream of gaseous nitrogen at 45°C. The residue was reconstituted with 500 µl of mobile phase and 10 µL aliquots were injected into the HPLC system.

Bioanalytical Method validation

United States Food and Drug Administration (USFDA) [19] guidelines for industry: Bioanalytical method validations were followed for the validation of the developed method.

System Suitability

The suitability of the system was performed by injecting six consecutive injections using a standard aqueous mixture equivalent to the MQC concentration of the calibration curve. The suitability of the system was performed at the beginning of the validation of the method and every day as the first experiment.

![Fig. 1: Chemical structures of Metoprolol (A) and Propranolol (B).](image-url)
Specificity/Selectivity:

The specificity of the LC-MS/MS method was established by selecting the standard blanks of different lots of commercially available human plasma. Ten different plots of plasma were examined for the specificity experiment. Out of every ten, seven lots of anticoagulant plasma, hemolytic plasma, a lipid plasma and a lot containing heparin as an anticoagulant were planned. It was found that all the human plasma plots studied did not exhibit significant interferences in drug and IS retention time (that is, the peak area in drug retention time in standard white samples was ≤ 20.00% of area of the drug in the extracted LLOQ sample, the ISTT retention time peak area in standard white samples was ≤ 5.00% of the ISTD area in the extracted LLOQ sample). Plasma lots were grouped and used as an empty array to prepare samples of calibration curves and quality control samples.

Linearity and Quality controls:

The linearity of the method was determined using a 1/x2 weighted least squares regression analysis of standard graphs associated with a standard ten-point curve. It was found that the three calibration curves tested in the course of validation were linear for the concentration of standards ranging from 1.555 to 538.254 ng/ml. The chromatography observed during the validation course was acceptable and the representative chromatograms of Blank Standard, Zero Standard (Standard empty with internal standard), Standard LLOQ, Standard ULOQ, HQC, MQC1, MQC2 and LQC were shown in the results.

Recovery:

Recovery for the analyte:

The% of average recoveries was determined by measuring the responses of the plasma quality control samples taken compared to the quality control samples taken at HQC, MQC1, MQC2 and LQC levels.

Recovery for the internal standard:

The% of average recoveries was determined by measuring the responses of the internal standard respectively in the samples extracted from the samples extracted.

Effect of the matrix:

The effect of the matrix for the LC-MS/MS method was evaluated using six different plots of human plasma subjected to chromatographic screening. With each lot of plasma, the concentrations of samples equivalent to LQC and HQC were prepared by Metoprolol and injected in triplicate at each level.

Accuracy and Precision:

The accuracy of the test was calculated as the absolute value of the relationship between the calculated mean values of the quality control samples and the respective nominal values, expressed as a percentage.

Precision was evaluated by% CV at different concentration levels corresponding to LLOQ, LQC, MQC2, MQC1 and HQC during the validation process. Precision and precision were determined within the lot and between the lots.

Stability

Stability experiments were performed to evaluate the stability of Metoprolol and the internal standard (Propranolol) in human plasma during sample preparation and sample analysis. To determine the stability of the upper part of the table, the quality control samples were determined during a 6-hour period stored at room temperature. The long-term stock solution and stability of the standard analyte and IS working solution were determined using a standard equivalent to the concentration of HQC, after a period of storage of 6 days at 5 ± 3 °C. Freezing and defrosting of the enriched quality control samples were determined after the three freeze-thaw cycles stored at -28 °C ± 5 °C. The stability of the automatic injector of the treated quality control samples was determined during a period of 28 hours and 22 minutes storing them in the autosampler maintained at a temperature of 5 °C ± 3 °C. For all experiments, the concentrations of metoprolol and propranolol were compared with the nominal values. Stability was assessed by comparing with newly added calibration standards and quality control samples. The average stability% for quality control standards should be between 85 and 115%.

Results and Discussion

Bioanalytical method development by LC/MS-MS:

Metoprolol and Internal standard, Propranolol was resolved by using various combinations of aqueous solutions and organic phases as methanol or acetonitrile in mobile phase. The paper describes the elution of Metoprolol and IS using 5mM Ammonium Formate pH 3.5 and Acetonitrile (15:85 % V/V) as mobile phase. Mobile phase flow rate was maintained at 1.00 mL/min. The column temperature was 40°C. The injection volume was 5µL. Metoprolol was eluted at 1.11±0.35
minutes and Propranolol at 1.20±0.48 minutes. Both the peaks were well resolved without any interference.

Liquid-liquid extraction (LLE) was used to prepare the sample in this work. LLE can be useful for producing a spectroscopically clean sample and preventing the introduction of non-volatile materials into the column and the MS system. Clean samples are essential to minimize ion suppression and matrix effect [20] in LC / MS / MS analyzes. Methyl-tert-butyl ether was found to be optimal, which can produce a clean chromatogram for a white plasma sample.

Analyte and IS recoveries were good and were consistent, accurate and reproducible. Therefore, the method has been shown to be robust in high performance bioanalysis. The choice of the appropriate internal standard is an important aspect to obtain an acceptable performance of the method, especially with LC / MS / MS, where the effects of the matrix can lead to mediocre analytical results. Several compounds have been studied to find an adequate IS, and finally it has been found that propranolol is suitable for the current purpose. Furthermore, its preservation behavior is similar to that of the target analyte. Clean chromatograms were obtained and no significant direct interference was observed in MRM channels in the relevant retention times. However, in the ESI, signal suppression or improvement may occur due to endogenous co-elution components of the sample matrix.

The potential for the co-extracted matrix component to influence the analyte and IS detector response was evaluated in six separate batches of K2EDTA human plasma and a lot of hemolyzed plasma. The aqueous standard equivalent to the concentration of the LQC level together with the expected concentration of the internal standard was added to the empty matrix subsequently extracted.

Fragmentation voltages tested were from 80 to 200 V, so as to reduce unwanted fragmentation. At 120 V mass spectrometric response of metoprolol and Propranolol showed least unwanted fragmentation with good response. The mass transition ion-pair was followed as m/z 268.15→115.90 for Metoprolol and 260.17→115.90 for Propranolol. Highest sensitivity was achieved for Metoprolol and Propanolol protonated molecular ions at [M+H]+ (m/z 268.160) and (m/z 260.17) respectively and m/z 115.90 and 116.20 were selected as quantifier ion peaks for metoprolol and Propanolol respectively after optimization.

Other parameters of mass spectrometry, ie the gas temperature, the gas pressure and the gas flow have been adjusted to obtain a maximum signal for metoprolol. The flow of nebulizing gas was adjusted to 13 l/min, the temperature of the drying gas to 315 °C and the capillary tension to 4250 V. The response of Metoprolol and Propranolol was measured by MRM in a positive ionization mode with collision energy of 25 V.

Validation of LC/MS-MS method:

The method has been validated according to the USFDA guidelines of the bioanalytical method [19]. The parameters generally examined in the validation process are selectivity, linearity and quality controls, matrix effect, recovery, precision and stability.

System Suitability:

The % CV of the retention times was found to be ≤ 0.42 and 0.34 for the analyte and IS respectively. The % CV of the peak area ratio (Analyte area/IS area) was found to be ≤ 3.44.

Specificity/ Selectivity:

No interference was found at the retention times of analyte and IS and no cross-interference between analyte and IS. Fig 2 shows the representative chromatograms of blank human plasma (Fig 2A); human plasma spiked with IS, Propranolol (Fig 2B); human plasma spiked with analyte and IS (Fig 2C). As it can be seen in Fig 2C, that no overlapping peaks were detected at Metoprolol and Propranolol retention times 1.10 minutes and 1.19 minutes respectively. The bioanalytical method proved to be specific and selective.

Linearity and Quality controls:

The linearity of the method was determined using a 1 / x2 weighted least squares regression analysis of standard graphs associated with a standard ten-point curve. A good linear relationship between the MPL / IS peak area ratio and the MPL concentration in the range of 1.555 to 538.254 ng / mL was shown. The average correlation coefficient (r2) was 0.9975 (n = 3). The mean equation of the calibration curve was y = 0.0453X + 0.0064 (n = 3) with slope 0.04538 ±1.45 and intercept of 0.00643 ±1.92. The least squares regression revealed that the relationship was linear, satisfying the acceptance criteria (r2≥0.990). The lower limit of quantification, which is the lowest standard level with a coefficient of variation of less than 20%, is 1.55 ng / mL with a signal to noise ratio of 36.214. The bioanalytical method has proved to be sensitive, allowing a precise quantification of concentrations starting from 1.5 ng / ml.
Fig. 2: The representative LC-MS/MS chromatograms obtained from (A) Blank human plasma sample, (B) Human plasma spiked with IS, Propranolol and (C) System suitability sample at 233.978 ng/ml of Metoprolol.
Table-1: Accuracy and Precision of the LC/MS-MS method.

<table>
<thead>
<tr>
<th>QC ID</th>
<th>HQC</th>
<th>MQC1</th>
<th>MQC2</th>
<th>LQC</th>
<th>LLOQ QC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nominal Concentration (ng/mL)</td>
<td>417.819</td>
<td>233.978</td>
<td>112.310</td>
<td>4.492</td>
<td>1.572</td>
</tr>
<tr>
<td>355.146 - 480.492*</td>
<td>198.881 - 269.075*</td>
<td>95.464 - 120.157*</td>
<td>3.818 - 5.166*</td>
<td>1.258 - 1.886*</td>
<td></td>
</tr>
</tbody>
</table>

Calculated Concentration (ng/mL)**

| Mean | 378.79 | 222.40 | 109.566 | 4.4088 | 1.4645 |
| SD | 13.198 | 6.966 | 4.748 | 0.182 | 0.093 |
| % CV | 3.518 | 3.130 | 4.373 | 4.148 | 6.435 |
| % Mean Accuracy | 90.66 | 95.05 | 97.56 | 98.15 | 93.16 |

Between Batch Precision and Accuracy (n=24)

| Mean | 378.7935 | 222.3961 | 109.566 | 4.4088 | 1.4645 |
| SD | 19.2123 | 9.7914 | 5.5872 | 0.2057 | 0.1085 |
| % CV | 5.07 | 4.40 | 5.10 | 4.67 | 7.41 |
| % Mean Accuracy | 90.66 | 95.05 | 97.56 | 98.15 | 93.16 |

*acceptance concentration range: The within and between batch precision for LQC, MQC and HQC samples should be ≤ 13.00 % and for the LLOQ QC, it should be ≤ 20.00 %.

** Average of 4 days values.

Table-2: Results of Matrix effect.

<table>
<thead>
<tr>
<th>QC</th>
<th>HQC</th>
<th>LQC</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. No.</td>
<td>Nominal Concentration (ng/mL)</td>
<td>Calculated Concentration (ng/mL)**</td>
</tr>
<tr>
<td>Mean</td>
<td>355.146 - 480.492*</td>
<td>3.818 - 5.166*</td>
</tr>
<tr>
<td>SD</td>
<td>17.3372</td>
<td>0.2255</td>
</tr>
<tr>
<td>% CV</td>
<td>4.84</td>
<td>5.58</td>
</tr>
<tr>
<td>% Mean Accuracy</td>
<td>90.66</td>
<td>95.05</td>
</tr>
</tbody>
</table>

*acceptance concentration range: The within and between batch precision for LQC, MQC and HQC samples should be ≤ 13.00 % and for the LLOQ QC, it should be ≤ 20.00 %.

** Average of six different plasma lots

Accuracy and Precision:

The within-run and between-run accuracy and precision of the analytical method were determined by six replicate analysis of Metropolot at four concentrations of QC samples (LLOQ: 1.572 ng/mL, LQC: 4.492 ng/mL, MQC-1: 233.978 ng/mL, MQC-2: 112.310 ng/mL and HQC: 417.819 ng/mL) in five individual batches on four different days. The % CV and % mean accuracy with-run for precision and accuracy ranged from 1.73 – 8.90 % and from 85.17 – 100.87 % respectively. The % CV and % mean accuracy between-run for precision and accuracy ranged from 4.40 – 7.41 and 90.66 – 98.15% respectively. All of precision and accuracy results (Table-1) were within the acceptance limit according to guidelines b USFDA for bioanalytical method validation. These data confirmed that this newly developed LC-MS/MS method has precision, accuracy and reproducibility to determine Metropolot. The chromatograms of all QCs were shown in Fig. 3.

Recovery and Matrix effect:

The recovery results showed highly consistent recovery coefficient in both analyte and IS. The mean recovery of LQC, MQC 1 and 2 and HQC were 72.78 %, 64.25 %, 70.27 % and 7019 % respectively for metropolot. Mean recovery value for the IS is 68.35 %. Additionally, matrix effect was an important factor for evaluation because biological matrix could affect the signal of metropolot in LC-MS/MS analysis. The% CV values of six different plasma lots were less than 5.6%, indicating that no coeluting substance influenced the ionization of analytes or IS. This result indicates that the extraction efficiency for the analytes using liquid-liquid extraction was satisfactory, consistent and independent of concentration. Table-2 shows the results of the matrix effect.

Stability

The stability of metropolot in human plasma and stock standard solution were tested and summarized in Table-3. Stability data were shown in the form of mean value obtained from 2 concentrations of QC levels (LQC and HQC, n=6). Metropolot and IS were proved to be stable in human plasma at specified storage conditions for post-preparative stability, re-injection reproducibility, bench top stability, freeze-thaw stability (three cycles), long-term stability (120 days) with the percentage difference and precision (%CV) were within an acceptable range of ±15%. Accuracy of the observed mean concentration was within 85-115% of their respective nominal concentrations. All of data were reflected satiations of sample handing and analysis. The stability of metropolot and IS in stock solution were evaluated for 30 days at -70°C ±5°C. We found that the stability results were within ± 2% of their observed peak areas at 30 days storage stock solution by respective peak area of freshly prepared stock solution. This indicated that metropolot and IS was stable in plasma during sample preparation process and storage conditions. Table-3 shows the stability data.
Table-3: Stability of metoprolol in different storage conditions.

<table>
<thead>
<tr>
<th>Stability</th>
<th>QC Level</th>
<th>Mean Measured concentrations (ng/ml); (n=6)</th>
<th>% Change</th>
<th>Precision (%CV)</th>
<th>Accuracy (%)</th>
<th>% Mean stability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Comparison sample</td>
<td>Stability sample</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Auto sampler</td>
<td>HQC</td>
<td>409.1053±9.314</td>
<td>387.526±11.805</td>
<td>-5.275</td>
<td>3.05</td>
<td>92.75</td>
</tr>
<tr>
<td></td>
<td>LQC</td>
<td>4.7722±0.354</td>
<td>4.6107±0.218</td>
<td>-3.384</td>
<td>4.73</td>
<td>102.64</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>396.993±5.594</td>
<td>401.387±6.685</td>
<td>1.107</td>
<td>2.66</td>
</tr>
<tr>
<td>Bench top</td>
<td>HQC</td>
<td>4.3517±0.183</td>
<td>4.6052±0.596</td>
<td>5.825</td>
<td>12.95</td>
<td>102.52</td>
</tr>
<tr>
<td>Wet extract</td>
<td>HQC</td>
<td>4.7722±0.354</td>
<td>4.6177±0.253</td>
<td>-3.238</td>
<td>5.48</td>
<td>102.80</td>
</tr>
<tr>
<td>at 5 ± 3°C</td>
<td>LQC</td>
<td>396.993±5.594</td>
<td>402.523±3.541</td>
<td>1.393</td>
<td>2.12</td>
<td>96.34</td>
</tr>
<tr>
<td>Wet extract at 25 ± 3°C</td>
<td>HQC</td>
<td>4.3517±0.183</td>
<td>4.3578±0.187</td>
<td>0.140</td>
<td>4.30</td>
<td>97.01</td>
</tr>
<tr>
<td></td>
<td>LQC</td>
<td>409.1053±9.314</td>
<td>393.637±5.902</td>
<td>-3.781</td>
<td>1.50</td>
<td>94.21</td>
</tr>
<tr>
<td>Dry extract</td>
<td>HQC</td>
<td>4.7722±0.354</td>
<td>4.4500±0.353</td>
<td>-6.752</td>
<td>5.69</td>
<td>99.07</td>
</tr>
<tr>
<td></td>
<td>LQC</td>
<td>396.993±5.594</td>
<td>397.739±3.378</td>
<td>0.188</td>
<td>1.85</td>
<td>95.19</td>
</tr>
<tr>
<td>Freeze-Thaw</td>
<td>HQC</td>
<td>4.3517±0.183</td>
<td>4.4640±0.306</td>
<td>2.581</td>
<td>6.87</td>
<td>99.38</td>
</tr>
<tr>
<td></td>
<td>LQC</td>
<td>405.0681±0.256</td>
<td>394.8936±0.254</td>
<td>-2.512</td>
<td>3.21</td>
<td>99.85</td>
</tr>
<tr>
<td>Short term</td>
<td>IS</td>
<td>4491.9±0.147</td>
<td>4.6112±0.149</td>
<td>2.657</td>
<td>2.54</td>
<td>96.62</td>
</tr>
<tr>
<td></td>
<td>HQC</td>
<td>495.55±0.657</td>
<td>496.982±0.201</td>
<td>0.289</td>
<td>1.01</td>
<td>100.20</td>
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<tr>
<td></td>
<td>LQC</td>
<td>401.0310±1.526</td>
<td>397.9668±3.320</td>
<td>-0.764</td>
<td>6.21</td>
<td>95.37</td>
</tr>
<tr>
<td></td>
<td>IS</td>
<td>496.982±3.210</td>
<td>491.253±4.201</td>
<td>-1.153</td>
<td>4.20</td>
<td>97.20</td>
</tr>
</tbody>
</table>

% Change = \( \frac{\text{Mean stability sample} - \text{Mean comparison sample}}{\text{Mean comparison sample}} \times 100 \)

Fig. 3: Representative chromatograms of (A) LLOQ at 1.572 ng/mL, (B) LQC at 4.492 ng/mL, (C) MQC-1 at 112.310 ng/mL, (D) MQC-2 at 233.978 ng/mL and (E) HQC at 417.819 ng/mL.

**Method Application:**

This LC-MS/MS method will be applied in clinical studies on hypertensive patients at Santhiram Medical College and general hospital, Nandyal, India and extent of absorption and other pharmacokinetic parameters of metoprolol from different oral formulations will be determined. Results will be available shortly.

**Conclusion**

The proposed LC-MS/MS method is rapid, sensitive and reproducible for the quantification of
metoprolol in human plasma with a wide linear dynamic range of 1,505-538,254 ng/mL. It was validated and met all the requirements according to the USFDA standards guidelines with high degree of accuracy and precision. Absence of matrix effects was adequately demonstrated. In addition, the stability study indicated that metoprolol was stable in plasma during sample preparation process and other storage conditions. The lower LLOQ, smaller plasma volume and shorter run time (<2 min) make our new method particularly suitable for use in routine bioanalytical and bioequivalence assay studies.

Acknowledgement

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